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ABSTRACT

Enamel-related gene products (ERPs) are detected in non-enamel tissues such as bone. We hypothesized that, if functional, ERP expression corresponds with distinct events during osteoblast differentiation and affects bone development and mineralization. In mouse calvariae and MC3T3 cells, expression profiles of enamel-related gene products (ERPs) correlated with key events in post-natal calvarial development and MC3T3 cell mineralization. Developing skulls from both *Amel*- and *Ambn*-deficient animals were approximately 15% shorter when compared with those of wild-type controls, and their sutures remained patent for a longer period of time. Analysis of *Amel*- and *Ambn*-deficient calvariae and calvarial osteoblast cultures revealed a dramatic reduction in mineralized nodules, a significant reduction in *Runx2*, *Sp7*, *Ibsp*, and *Msx2* expression, and a reduction in *Alx4* in *Amel*-deficient calvariae vs. an increase in *Alx4* in *Ambn*-deficient calvariae. Analysis of these data indicates that ERP expression follows defined developmental profiles and affects osteoblast differentiation, mineralization, and calvarial bone development. We propose that, in parallel to their role in the developing enamel matrix, ERPs have retained an evolutionary conserved function related to the biomineralization of bones.

KEY WORDS: amelogenin, ameloblastin, osteoblast, calvaria, evolution, suture.

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Expression and Function of Enamel-related Gene Products in Calvarial Development

INTRODUCTION

The mineralized tissues bone, dentin, cementum, and tooth enamel play quintessential roles in the life of organisms, acting as guardians of the brain and powerful effectors of mastication. Their formation and maintenance are tightly controlled by several phosphorylated and repeat-element-containing matrix proteins, which are encoded by a cluster of secretory phosphoprotein-encoding genes (SCPP) located on chromosome 4 (Huq *et al.*, 2005). One half of these proteins is associated with the formation of bone and dentin, counting bone sialoprotein (BSP), osteopontin (OPN), dentin sialophosphoprotein (DSPP), dentin matrix protein (DMP), and matrix-extracellular phosphoglycoprotein (MEPE) among its members. The other half consists of the enamel-associated proteins ameloblastin (AMBN), amelogenin (AMEL), amelotin (AMTN), and odontogenic ameloblast-associated protein (ODAM). Besides their affiliation with the same gene cluster, SCPP proteins contain several sequence elements, including internal repeats, acidity of select domains, phosphorylation, and negative charge, that predispose them to promote mineral nucleation through calcium ion sequestration, or to control crystal growth by adsorption onto growing crystal surfaces, or both (Addison and McKee, 2010).

The first study pointing to common gene products in enamel and other mineralized tissues was based on a monoclonal antibody generated against a hamster tooth germ (Inai *et al.*, 1993). Subsequently, other studies have blurred the distinction between enamel-type and bone/dentin-type mineralized tissues, highlighting expression of amelogenin (Amel) in dentin, cementum, periodontal ligament, long bone, brain, and soft tissues (Deutsch *et al.*, 2006; Li *et al.*, 2006; Ye *et al.*, 2006; Zeichner-David *et al.*, 2006; Haze *et al.*, 2007) and Ambn expression in dentin, cementum, pulp, and cranial bones (Fong *et al.*, 1998; Spahr *et al.*, 2002; Hao *et al.*, 2005; Nunez *et al.*, 2010).

The ubiquitous expression of SCPPs in multiple mineralized tissues in conjunction with similar but distinct functions suggests that SCPP proteins collaborate to control and fine-tune the growth of craniofacial mineralized tissues. Here, we have conducted studies to define the role of ERPs in craniofacial mineralized tissue development. First, ERP expression in calvarial bone and tooth enamel development was mapped and correlated with key events in calvarial mineralization. To test the role of 2 key ERPs in osteogenesis, we determined the effects of AMBN and AMEL on mineralization markers, mineral formation, and calvarial development using a diet-independent cell

culture model in tandem with mutant mouse models. Together, these studies provide a re-evaluation of the roles of ERPs in mineralized tissue development.

MATERIALS & METHODS

Animals and Cell Culture

Post-natal CD1 mice age 3, 10, 20, and 35 days ($n = 48$) (Charles River Laboratories, Wilmington, MA, USA), as well as B6;129 wild-type, *Amelx*^{-/-} (Gibson *et al.*, 2001), and *Ambn*^{-/-} ($n = 48$) (Fukumoto *et al.*, 2004) stage-matched mice were handled in accordance with the University of Illinois at Chicago Use of Animals in Research protocol. Mouse calvaria-derived MC3T3-E1 cells (CRL-2593) subclone 4 were maintained in α -minimum essential medium (Gibco BRL, Gaithersburg, MD, USA). Primary calvarial osteoblasts were obtained from calvariae of P1 mice (*Amelx*^{-/-}, *Ambn*^{-/-}, wild-type). For osteoinduction, primary calvarial cells were cultured on 6-well culture plates containing Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA), 50 μ g/mL of ascorbic acid, and 3 μ M of beta-glycerophosphate.

Alcian Blue/Alizarin Red Staining

Three CD1 mice *per* time-point (P3-35), and P2 B6;129 and ERP mutant mice were fixed and dehydrated with ethanol and acetone, stained with alcian blue and alizarin red S for 2 days, immersed in 0.5% potassium hydroxide solution, and stored in 80% glycerol.

RT-real-time PCR

Total mRNA was extracted from calvariae and molars of CD 1 mice at pre-determined developmental stages or from MC3T3 cells after 7, 14, 28, and 42 days' culture in mineralization medium. To quantify mRNA expression levels, we performed RT-real-time PCR using sequence-specific SYBR Green primers (Appendix Table 1) and the ABI Prism 7000 detection system (Applied Biosystems, Carlsbad, CA, USA). Samples were normalized with β -actin. Relative expression levels were calculated by the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). For mRNA expression studies, day 0 in culture and P3 in calvariae were used as baseline ($= 1$), and β -actin was used as an endogenous control. All PCR products were sequenced at the UIC DNA facility for sequence verification.

Immunohistochemistry

Calvarial vaults from CD1 mouse heads at age P6 were fixed, decalcified, paraffin-embedded, and sectioned. Sections were processed and incubated with ERP protein primary antibodies (Appendix Table 2) at a 1:50 dilution as reported (Lu *et al.*, 2013).

Western Blot Analysis

Calvariae from CD1 mice aged P2, P10, and P20 were ground under liquid N₂. Aliquots of calvarial protein extracts were subjected to Western blotting (primary antibody dilutions listed in

Appendix Table 2). Tooth proteins from extracted maxillary molars of P3 mice were used as positive controls.

In vitro Mineralization Assay

In vitro mineralization assays (alizarin red) were performed on MC3T3 cultures just before the medium was switched to mineralization medium (wk 0) and at each subsequent interval (wks 1, 2, 4, and 6).

Statistical Analyses

Analyses were performed in triplicate for 3 independent experiments. Kruskal-Wallis analysis of variance was used to determine the mean difference, and independent *t* tests were used to determine the pair-difference at $p = .05$.

RESULTS

Enamel-related Gene Products' (ERPs) Expression in Mouse Calvariae

To determine whether occurrences of ERPs in tissues outside of enamel follow concise developmental patterns, we mapped longitudinal ERP expression levels in correlation with calvarial development stages from days P3-P35 (Fig. 1A). The bony bridge of the posterior frontal suture began to form in P20 animals and closed completely on day 35 (Fig. 1A). mRNA expression levels of ERPs, *Amel*, *Ambn*, *Enam*, *Amtn*, *Odam*, *Mmp20*, and *Klk4* were detected in calvarial tissues at all stages of development. During calvarial development, ERP profiles changed substantially (Fig. 1B), and expression of ERPs gradually decreased from P3 to P35, with the exception of *Ambn* and *MMP20*, which peaked at P20 and then decreased gradually.

MC3T3 pre-osteoblasts proliferate, differentiate into osteoblasts, and exhibit signs of mineralization in long-term cell culture (Quarles *et al.*, 1992). Here we used the MC3T3 long-term cell culture model to investigate whether ERPs display specific expression profiles during osteoblast differentiation and mineralization. RT-real-time PCR analysis indicated that all studied ERPs (*Amel*, *Ambn*, *Enam*, *Amtn*, *Odam*), as well as the enzymes *Mmp20* and *Klk4*, were expressed during the entire 42 days of culture. To compare ERP expression profiles with key stages of MC3T3-related mineral formation and osteoblast differentiation in culture, we assessed mineralization levels using an *in vitro* mineralization assay (Fig. 1C) and identified benchmarks of osteoblast differentiation using *Coll1A1*, *Ibsp*, and *Ocn* mRNA expression levels as reference points. ERPs exhibited distinct expression patterns and profile changes during key stages of matrix mineralization and differentiation of MC3T3 cells (Fig. 1D). Specifically, *Amel* and *Mmp20* expression profiles revealed a bi-phasic pattern (Fig. 1D), corresponding to the onset of mineralization and the peak expression of *Coll1A1* and *Ibsp* (28 days of culture, Fig. 1E). In contrast, *Ambn*, *Amtn*, *Odam*, and *Klk4* expression levels peaked at day 7 (Fig. 1D), corresponding to the early differentiation of MC3T3 cells (Fig. 1C), and then gradually decreased. Data are from 3 independent studies, which yielded essentially identical findings.

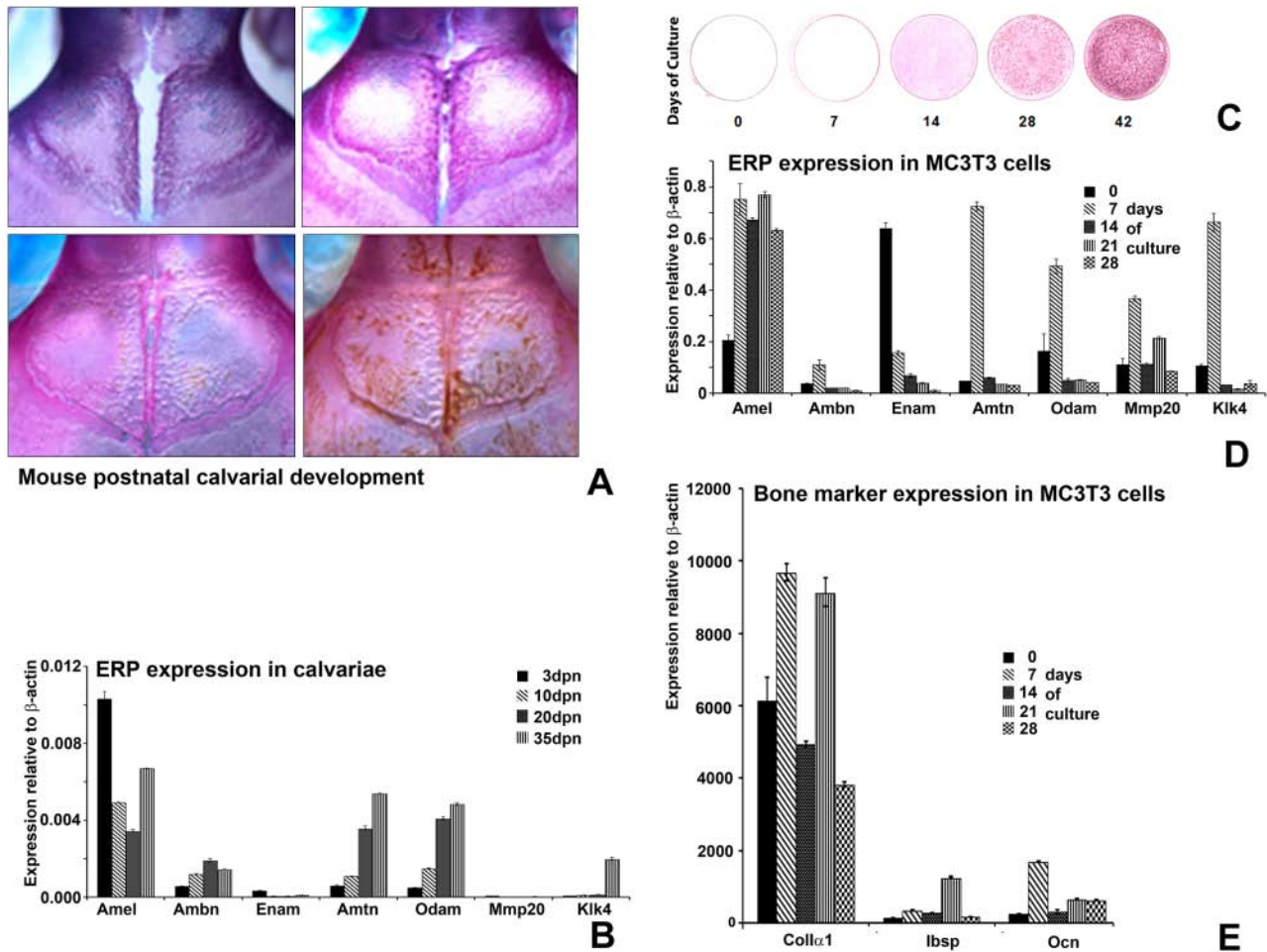


Figure 1. Expression profiles of enamel-related gene products (ERPs) correlated with post-natal calvarial development and MC3T3 cell mineralization. **(A)** Mouse calvariae visualized by Alcian blue/Alizarin Red S staining. The red staining in calvarial preparations represents developing calvarial mineralized tissues. ERP mRNA expression relative to endogenous control (β -actin) mRNA was detected at all stages of development and levels of most ERPs expression, with the exception of *Ambn*, gradually decreased with animal age **(B)**. **(C)** Alizarin red *in vitro* mineralization assay illustrating stages of mineralization during MC3T3-E1 cell culture over a period of 42 days. **(D)** Time-course of ERP mRNA expression levels relative to β -actin in MC3T3-E1 cells as revealed by real-time RT-PCR. **(E)** Matched real-time RT-PCR analysis of bone marker mRNA expression levels relative to β -actin *Amel*- (A) and *Ambn*- (B) deficient mice in MC3T3-E1 cells.

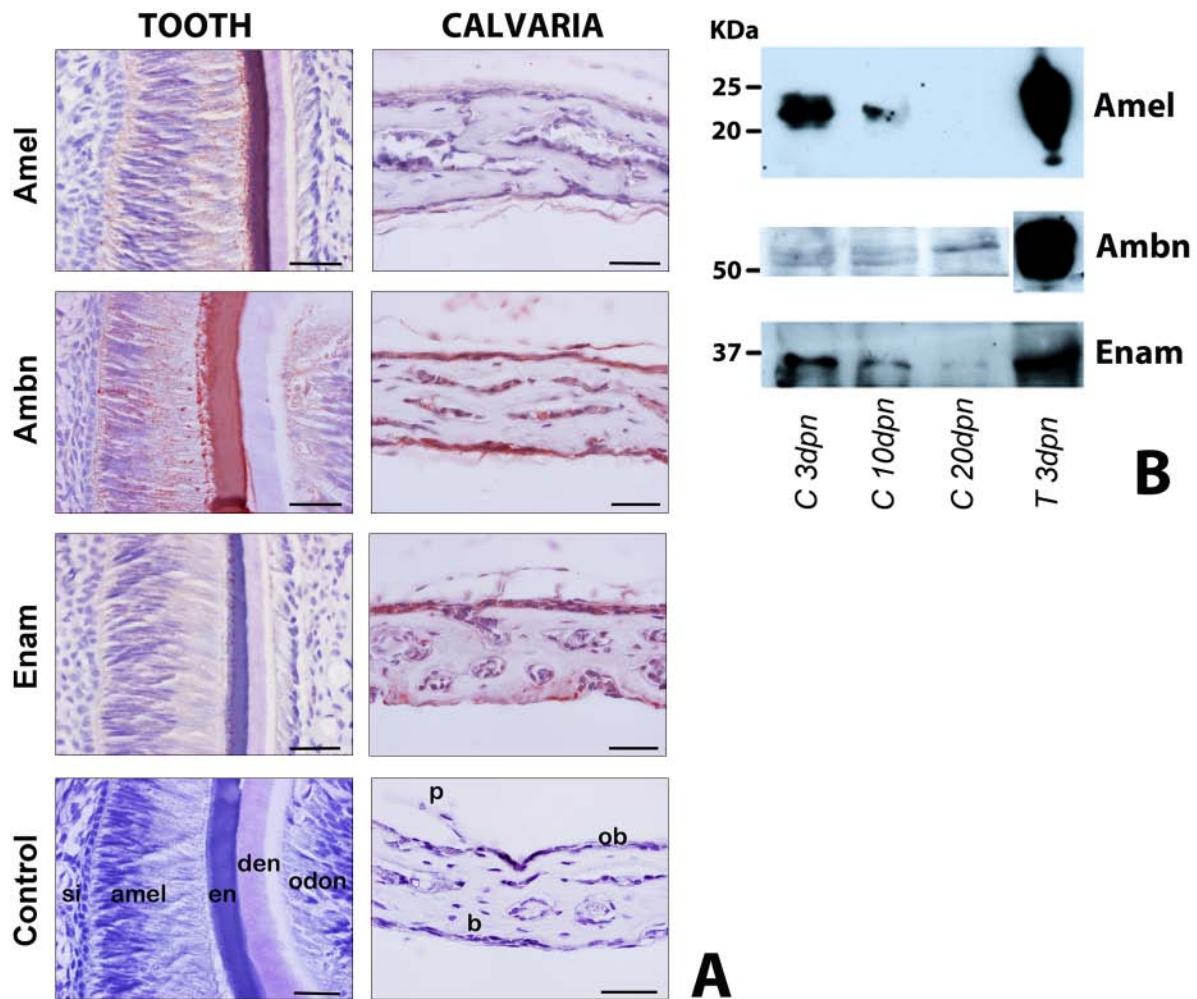
Immunohistochemistry and Western Blotting of ERPs in Developing Calvariae

Amelogenins constitute 90% of the enamel protein matrix, whereas collagen 1a1 contributes 90% of the bone protein matrix. To determine whether ERPs contribute significantly to the non-collagenous calvarial bone protein matrix, we compared ERP protein and mRNA expression levels in developing mouse calvariae and teeth. First, *Amel*, *Ambn*, and *Enam* were immunohistochemically detected in calvarial tissues from P6, mainly in the bone matrix and periosteum (Fig. 2A, right panel). Weak levels of reactivity were also documented in osteoblasts, osteocytes, and periosteal cells (Fig. 2A, right panel). *Amel*, *Ambn*, and *Enam* were localized in ameloblasts and enamel matrices (Fig 2A, left panel). Using Western blotting, we detected all 3 ERPs (*Amel*, *Ambn*, *Enam*) in P3, P10, and P20 calvariae as well as in T3 teeth, with the exception of *Amel* in P20 calvariae (Fig. 2B). Protein levels for all 3, *Amel*, *Ambn*, and *Enam*, were higher in P3 calvariae than in

calvariae from later stages of post-natal development, while ERP levels in T3 teeth exceeded those found in P3 calvariae by approximately 60- to 1,000-fold (calculated after adjustment for the 1:20-1:40 pre-dilution of enamel proteins in this Western blot; Fig. 2B). RT-real-time PCR teeth/calvariae expression comparisons revealed high ERP levels in 3dpn teeth when compared with calvariae, with higher *Amtn*, *Klk4*, *Odam*, and *Amel* expression levels in calvariae than other ERPs (Fig. 2C).

Amel and Ambn Affect Developing Skull Size and Suture Closure

In developing calvarial bone matrices and cultured MC3T3 cells, ERP levels were well beyond detection threshold, stage-specific, and time-dependent. These findings prompted us to determine whether the loss of key ERPs in calvarial cells would affect skull growth (Fig. 3). Phenotype penetrance was 100%, and an interfrontal bone in *Amel*-deficient mice was present in



Relative ERP mRNA expression in teeth VS calvaria

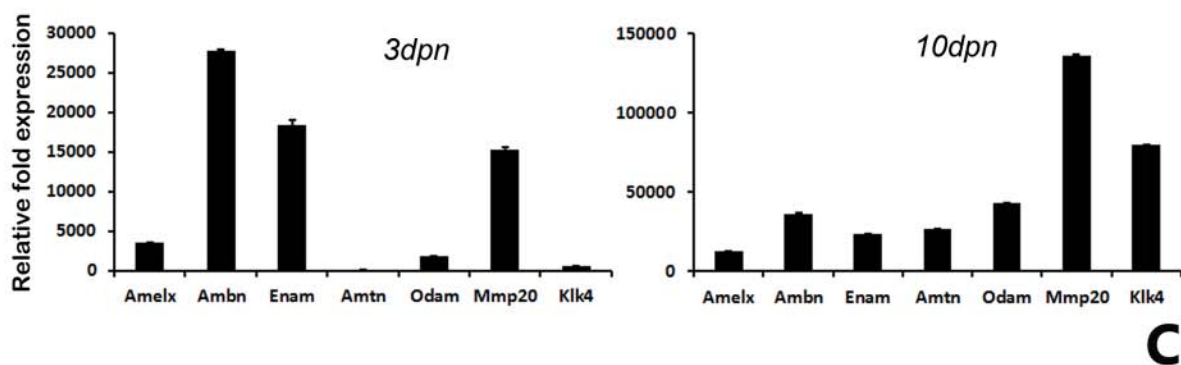


Figure 2. ERP expression comparison between post-natal developing tooth organs and calvariae. **(A)** Positive immunoreaction (red) for amelogenin (Amel), ameloblastin (Ambn), and enamelin (Enam) in ameloblasts, enamel matrix, bone matrix, and periosteum. Scale = 100 μ m. **(B)** Western blot detection of amelogenin (Amel), ameloblastin (Ambn), and enamelin (Enam) in calvarial bone extracts at post-natal days 3, 10, and 20 and in developing tooth organs at post-natal day 3. Note that, in this Fig., tooth extracts were diluted at a 1:20 ratio (Ambn and Enam) or a 1:40 ratio (Amel) dilution relative to calvarial extracts, for visualization of expression levels on the same blot. **(C)** Relative ERP mRNA expression levels between developing calvariae and teeth at post-natal days 3 and 10. Δ Ct were normalized to β -actin, and $\Delta\Delta$ Ct was performed to calculate fold changes in expression values relative to wild-type calvaria. si = stratum intermedium, amel = ameloblasts, en = enamel, de = dentin, od = odontoblasts, p = periosteum, b = bone, ob = osteoblasts.

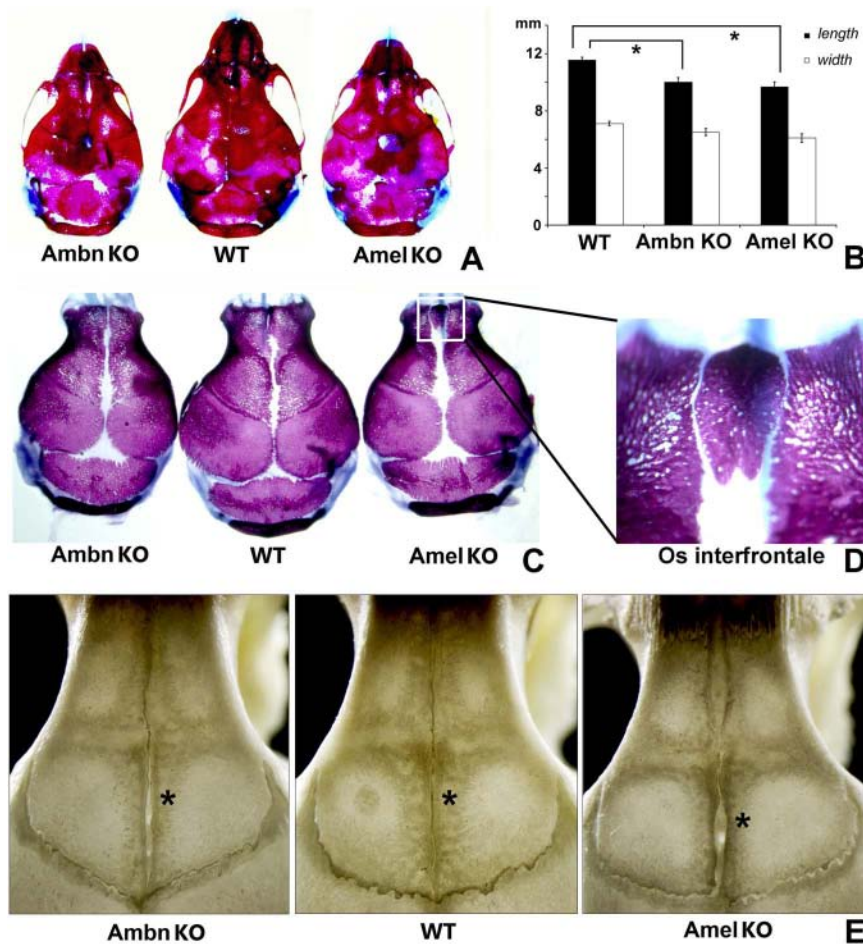


Figure 3. Comparison of skull dimensions and features among *Ambn*-deficient (*Ambn KO*), wild-type (WT), and amelogenin-deficient (*Amel KO*) mice. **(A)** Whole-skull comparison among representative skulls; **(B)** length and width comparison among *Ambn KO*, *Amel KO*, and WT mice; **(C)** calvarial vaults after removal of the cranial base; **(D)** pronounced interfrontal bone (*Os frontale*) in *Amel*-deficient mice; and **(E)** frontal suture patency in *Amel KO*, *Ambn KO*, and WT mice. * $p < .05$.

40% of the animals. Both skull length and width of P2 *Ambn*- and the *Amel*-deficient mice were significantly reduced compared with those of wild-type controls (16.2% *Ambn*^{-/-} and 13.4% *Amel*^{-/-} length reduction, $p < .05$; Figs. 3A, 3B). Moreover, suture closure was delayed in *Ambn*- and *Amel*-deficient animals (Fig. 3C), and an interfrontal bone was frequently found between the frontal bones of *Amel*-deficient mice (Fig. 3D). The interfrontal suture remained patent in 60 dpn in *Amel*^{-/-} and *Ambn*^{-/-} mice, and frontal bone length in *Amel*^{-/-} mice was substantially reduced (Fig. 3E).

Effects of *Amel* and *Ambn* on Osteoblast Differentiation

To investigate whether changes in skull size and mineralization were directly affected by ERP function and were independent of feeding behavior in ERP mutant mice, we assayed osteoblast mineralization and key mineralization marker gene expression. There was a significant reduction of *Ibsp* mRNA expression in *Amel*-deficient calvariae, while *Sp7* was reduced in

Ambn-deficient calvariae when compared with calvarial mRNA of wild-type littermates ($p < .05$, Figs. 4A, 4B). The suture-patency-related transcription factor *Msx2* was reduced in calvariae of both *Amel*- and *Ambn*-deficient mice, while *Alx4* was reduced in *Amel*-deficient mice and increased in *Ambn*-deficient mice. *Twist1* was reduced only in *Ambn*-deficient mice, while *Fgf2* was increased in *Amel*-deficient mice and not in *Ambn*-deficient mice (Figs. 4C, 4D). There was a highly significant 5- to 10-fold reduction in *Runx2*, *Sp7*, and *Ibsp* bone marker mRNA expression levels in *Amel*-deficient calvarial osteoblasts ($p < .05$, Fig. 4E) and a highly significant approximately 50% reduction in *Runx2*, *Sp7*, and *Ibsp* expression in *Ambn*-deficient calvarial osteoblasts ($p < .05$, Fig. 4F). Mineral nodule formation in *Amel*- and *Ambn*-deficient calvarial osteoblasts after 4-week culture was severely reduced (Fig. 4G).

DISCUSSION

The present study was designed to address 3 aspects relative to the presence of ERPs in tissues outside of enamel: (i) ERP expression levels in bone, (ii) correlation of ERPs in developing calvarial bone and in differentiating pre-osteoblasts with key events in mineralization and differentiation, and (iii) effects of calvarial osteoblast ERPs on mineralization behavior and bone marker gene expression (Diekwisch, 2011). To address these questions, we assessed ERP expression levels in developing

MC3T3 cells and calvarial osteoblasts and compared them with ERP expression levels in developing tooth enamel. To investigate whether ERPs were of functional relevance for bone development, we cultured osteoblasts from wild-type and *Amel*- and *Ambn*-deficient mice and examined them for mineralization potential and bone marker gene expression. In addition, skulls of *Amel*- and *Ambn*-deficient mice were analyzed.

Our study revealed that all ERPs studied here were detected in calvarial tissues and MC3T3 cells, and appeared to correlate with key time-points in osteoblast differentiation and mineralization. ERP expression patterns in MC3T3 osteoblast long-term culture peaked either at day 7 (*Ambn*, *Enam*, *Amtn*, *Odam*, and *Klk4*) or at day 28 (*Amel* and *Mmp20*). Based on parallel studies, these time-points correlated with either the early differentiation of osteoblasts, as indicated by a rise in *Coll1*, *Ibsp*, and *Ocn* at day 7, or with the beginning of matrix mineralization at day 28. Analysis of these data indicates that *Ambn*, *Enam*, *Amtn*, and *Odam* expression peaks are functionally correlated with events in osteoblast differentiation, while *Amel* might play

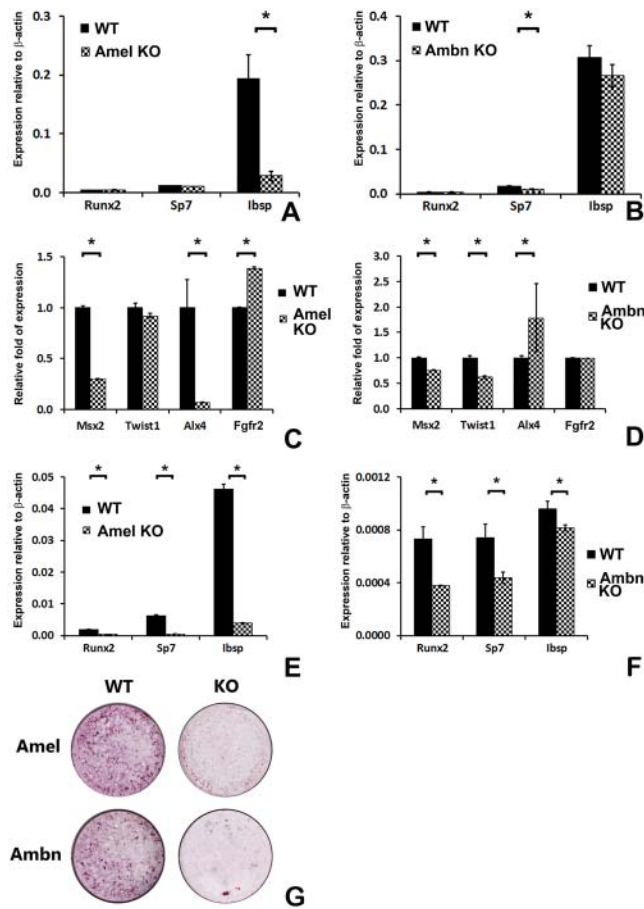


Figure 4. Bone transcription factor and marker expression and mineralization levels in calvariae and calvarial osteoblasts from ERP mutant mice. Bone marker RT-real-time PCR of calvariae (A,B) from *Amel*- and *Ambn*-deficient mice, suture-patency-related gene (C,D), and calvarial osteoblasts from *Amel*- (E) and *Ambn*- (F) deficient mice. *Msx2* expression was reduced, while *Alx4* expression showed different expression levels in calvariae from *Amel*- (C) and *Ambn*- (D) deficient mice. Note the significant reduction in bone marker and transcription factor gene expression levels, e.g., *Runx2*, *Sp7*, and *Ibsp*, in calvarial osteoblasts after loss of *Amel* and *Ambn* (E,F). (G) *In vitro* mineralization assay of primary calvarial osteoblasts from *Amel*- and *Ambn*-deficient mice compared with those of wild-types. **p* < .05.

a role in bone matrix mineralization. According to our immunoreactions, ERPs were detected not only in the bone matrix but also in the surrounding periosteum, suggesting that they might affect calvarial growth at both locations.

In response to the question about functional relevance of ERP expression in calvarial osteoblasts, our study revealed that several bone-related parameters were significantly reduced in both *Amel*- and *Ambn*-deficient models when compared with those in wild-type controls, including: (i) *Runx2*, *Sp7*, and *Ibsp* expression levels; (ii) osteoblast mineral nodule formation; and (iii) size of developing calvariae and suture closure. Together, these findings illustrate that both *Amel* and *Ambn* ERPs exhibit highly visible effects on bone matrix mineralization and bone marker gene expression. The concept that ERPs are involved in

osteoblast differentiation or bone matrix mineralization is supported by several previous studies related to the role of *Amel* in the modulation of mineralized tissue homeostasis, including activation of osteoclastogenesis (Hatakeyama *et al.*, 2006), modulation of cementogenesis (Swanson *et al.*, 2006), and bone marrow stem cell proliferation (Huang *et al.*, 2010). Recent studies suggested that *Ambn* binds CD63 and may act through integrin β 1 and C Src kinase inhibition to promote osteogenic differentiation (Iizuka *et al.*, 2011; Zhang *et al.*, 2011). Unique dysregulation profiles of major suture-patency-regulating genes such as *Msx2*, *Twist1*, *Alx4*, and *Fgfr2* (Melville *et al.*, 2010) in *Amel*- vs. *Ambn*-deficient mice suggest that individual ERPs affect individual transcription factor cascades to affect downstream effects on suture morphogenesis.

Five of the ERP genes studied here, *Ambn*, *Enam*, *Amtn*, *Odam*, and *Amel*, are members of the secretory calcium-binding phosphoprotein (SCPP) gene cluster of evolutionarily related skeletal mineralization molecules (Kawasaki and Weiss, 2008). According to our analysis, individual ERPs displayed 20% to 40% similarity with other SCPP family members, and bone proteins such as IBSP and OPN had more negatively charged amino acid residues than ERPs, suggesting that bone proteins may play a greater role in the regulation of crystal growth regulation (nucleation and/or inhibition) than ERPs. However, based on their sequence homologies, expression pattern overlap, and functional similarities, we suggest that all SCPP-derived proteins including ERPs contribute to the functional complexities of developing bone and other mineralized tissues. Evidence presented in this paper indicates that even after acquiring novel functions in enamel matrix organization, ERPs have retained their functional significance in bone development and homeostasis. Recent reports have shown a molecular decay of enamel matrix protein genes in turtles and other edentulous amniotes (Meredith *et al.*, 2013). Analysis of these data suggests that the presence of enamel proteins in bone and other mineralized tissues may not be essential for the survival of organisms, but may simply fine-tune the biochemical make-up of the mineralization scaffold of bones in tooth-bearing vertebrates.

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